Final report on the OTKA 69279 project.

In the eukaryotic cells, the cytoplasmic complex of the Importin-α and Importin-β proteins binds the cargo proteins by their nuclear localization signal (NLS), and carries them into the nucleus. There the GTP-bound Ran protein dissociates the complex liberating the NLS cargo. Beside the nucleo-cytoplasmic transport, the Importin-α-/Importin-β/NLS/RanGTP cycle plays important roles in other processes as well, e.g. in the early embryogenesis of *Drosophila melanogaster*.

There are three clades of Importin-α proteins (I-α1, I-α2, I-α3) coded by paralogous genes in *Drosophila*. The three Importins-α exert partly overlapping functions (GOLDFARB et al., 2004, Trends Cell Biol. 14, 505-514): I-α3 plays a decisive role in the nuclear protein import, while I-α1 and I-α2 are mainly active in the spermiogenesis and oogenesis, respectively. Previously, we were the first to describe the role of I-α2 in the formation of the ring canals during *Drosophila* oogenesis (Gorjánácz et al., 2002, Dev.Biol. 251, 271-282). In the present project the role of Importin-α2 in the early embryogenesis of *Drosophila melanogaster* was investigated. The experimental work was started in three directions:

1. functional role of the phosphorylation of !-α2;
2. interaction between the *importin-α2* and *importin-β* genes;
3. identification of genes interacting with the *importin-α2*^{D14} null-mutant (*D14*).

The <u>functional role of phosphorylation</u> was studied in *importin-α2* transgenic constructs where the potentially phosphorylated serines (S) were exchanged for the non-phosphorylateable alanine (A) or glutamic acid (E), the latter modelling the permanent phosphorylation. The UAS-constructs were transformed into *Drosophila*, and expressed by the *nos-GAL4* driver on a *D14/D14* homozygous mutant background. As we found, the S70A and S80A transgenes rescued the lethality of the embryos derived from *D14/D14* females while S37A, S56A and S65E were unable to rescue the maternal effect lethality. As the 37th and 56th positions are in the I-β-binding domain, our attention was focused on the interaction between I-α2 and I-β.

To identify <u>new genes interacting with importin–α2</u>, we screened the NigFly (Kyoto) collection of gene-silencing (RNAi) transgenic stocks as well as the DrosDel genome-wide series of overlapping deficiencies. The UAS-RNAi transgenes and the deletions were tested on the *D14/+* sensitized heterozygous background supposing that the genetic interaction would significantly decrease the fertility of *D14/+* females. After screening more than a thousand UAS-RNAi connstructs driven by the *nos-GAL4* driver, we found that the *Rpl7* gene coding for a structural protein of the large ribosomal subunit showed strong interaction with the *D14/+* background. Similar interaction was found with the *Nup358* gene, a constituent of the nuclear pore complex, identified with the deletions on the 3L chromosome arm. Despite these new results, we concentrated our efforts on the promising interaction between I-α2 and I-β (see below).

In <u>the investigation of the Importin-α2/Importin-β interaction</u>, we started out from the previous observation of ERDÉLYI and his co-workers (1997, Acta Biol. Hung. 48, 323-338) of the specific interaction between the null mutant *importin-α2*^{D14} and the *Ketel*^{RE34} mutant allele of *importin-β*: namely, early development of the syncytial embryos derived from *D14 +/+ Ketel*^{RE34} mothers, was blocked after a few nuclear divisions. *Ketel*^{RE34} (*RE34*) is a recessive lethal revertant of the dominant

female-sterile mutant *Ketel*^{D}. The strong interaction between *D14* and *RE34* is specific to the *RE34* allele as other *Ketel*^{D} revertants/null mutants do not show it.

We studied the maternal interaction between the *importin-α2* and *importin*-β genes with the help of genetic crosses, confocal microscopy and biochemical techniques. The results are summarized as follows:

a.) Lethality of the embryos from the *D14/RE34* mothers („maternal lethality") can be rescued by the wild-type transgenic cDNAs of both genes proving that the lethality is not a consequence of some background mutations.

b.) We tried UTR^{-} *importin-α1. -α2* and *–α3* transgenes whether they could rescue the maternal lethality of the embryos. Out of these transgenes producing the proteins at an equal rate, only *importin-α2* could rescue the maternal lethality. This result is the first to prove that I-α2 has a central, paralog-specific role in the assembly of the mitotic spindle which can not be replaced by I-α1 or I-α3.

c.) By making use of our *importin-α2* mutants deficient in the functional domains, we showed that the maternal interaction is realized through the NLS-binding site.

d.) With the help of immuno-staining (α-tubulin, centrosomin, lamin) and confocal microscopy, we found that the development of the lethal embryos is blocked in the metaphase of the first few nuclear divisions because of the failure of the mitotic spindles. The spindles showed various abnormalities: they were of larger than normal size, of abnormal shape, and contained more tubulin than wild-type spindles; the number of the centrosomes and the structure of the nuclear envelope were frequently abnormal, too.

e.) By co-immunoprecipitation and Western blotting we showed that the wild-type (but not the NLS^{-} mutant) I-α2 is able to bind at least three NLS-bearing mitotic proteins (ISWI, CP190, laminDm0).

f.) In GST-pulldown experiments we found that the RE34 mutant protein binds both RanGDP and RanGTP with higher affinity than that of the wild type I-β. This suggests that the I-α2/RE34/NLS complex can dissociate at a lower than normal RanGTP concentration.

g.) DNA sequencing showed that, in addition to the original P446L mutation causing the dominant female sterility, *Ketel*^{RE34} has a second-site D725N mutation probably responsible for the reversion of the dominant phenotype. Computer modeling showed that the conformation of the RE34 protein is more open than that of the wild-type which can increase the accessibility of the Ran binding site. These results are mainly due to the work of Erika Virágh, Tamás Szlanka and Mátyás Gorjánácz.

The above results and results from other laboratories suggest the following model:

In the *Drosophila* embryo there is no zygotic gene expression during the first 3 hours past fertilization, development of the early embryo is based on the maternal factors stored in the egg. The number of nuclei is doubled in every mitotic cycle, and the number of the *de novo* assembled spindles doubles accordingly. This can be explained by supposing that at the beginning the spindle assembly factors (SAFs) are bound in an inactive state, from which they are liberated gradually according to need. The previous studies mainly done in the *Xenopus* and *Drosophila* systems suggest the SAF proteins are stored bound through their NLS to the I-α/ I-β complex. The concentration of RanGTP is low in the cytoplasm but high in the vicinity of the chromosomes and chromatin. Therefore, the I-α/ I-

β/SAF complex dissociates near to the chromosomes, and the mitotic spindle is assembled of the liberated SAF proteins. Our results show that the SAFs bind to the I-α2/ I-β complex, and in this process the I-α2 plays a paralog-specific role, at least in the fruit fly. The formation of the complex needs the stoichiometric ratio of the constituents and the proper binding capacity for RanGTP. The mutant combinations we studied have a decreased SAF binding capacity, therefore the SAF proteins are freed at a higher than normal ratio leading to abnormalities in the formation and functioning of the mitotic spindles.

We summarized the above results in a publication submitted to Genetics (E.Virágh, M.Gorjánácz, I.Török, T.Eichhorn, S.Kallakuri, T.Szlanka, I.Kiss, B.M.Mechler, 2010: The fine-tuned cooperation between Importin-α2 and Ketel in spindle assembly during early *Drosophila* nuclear divisions.) After successfully finishing the additional experiments suggested by the reviewers, we are re-writing the text, and send it back to Genetics during this autumn.

Previous results were shown on posters at the VIII. Hungarian Genetics Congress (Nyíregyháza, 2009) and the 21st European Drosophila Research Conference (Nice, 2009).